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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
   D. Collen Research Foundation, C/O Dienst Prof. Collen, Campus Gasthuisberg, Herestraat 49, 3000 Leuven
   Represented by Prof. Collen, Chairman
   Patents ADP number *(if you know it)* 8508327001
   If the applicant is a corporate body, give the country/state of its incorporation: Belgium

4. Title of the invention
   HAEMOSTASIS

5. Name of your agent *(if you have one)*
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   D. Collen Research Foundation
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Signature: Prof. D. Collen, Chairman

Date: 16.01.03

12. Name and daytime telephone number of person to contact in the United Kingdom

Prof. D. Collen
Tel. 020 72449405 fax 020 72449406

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HAEMOSTASIS AND THROMBOPOIESIS

FIELD OF THE INVENTION

This inventions is relates to the control of the primary hemostasis and the modulation of platelet number and platelet function in a subject. More specifically the invention relates to use of pituitary adenylyl cyclase activating peptide (PACAP), its derivatives, mimetics or inhibitors or a platelet receptor, VPCA1, to modulate the primary haemostasis or thrombopiesis.

BACKGROUND OF THE INVENTION

The present invention relates to a new method for prevention and treatment of either thrombosis or bleeding based on administration of pituitary adenylyl cyclase activating peptide (PACAP) mimetics or inhibitors respectively.

The initial response to interruption of continuity of a blood vessel is defined as primary haemostasis. Platelets play a major role in the pathophysiology of primary haemostasis. The clinical importance of platelets became first obvious when thrombocytopenic patients who later on were diagnosed as having immune mediated thrombocytopenia (ITP) had purpura. Platelets participate in haemostasis by sealing vascular injuries and by fostering the process of blood coagulation. Not only the number of the platelets is important (thrombocytopenia for whatever reason) but also their intrinsic function upon activation: platelet shape change, adhesion, aggregation and secretion are prerequisites for normal haemostasis. Congenital or acquired disorders interfering with one of its function can lead to mild to even severe bleeding problems.

Prevention and treatment of bleeding in patients with thrombocytopenia or thrombocytopathia is therefore based on platelet transfusion or medication interfering with platelet number and/or function.

Platelets play also a role in the development of arterial thrombosis. Disruption of the endothelial cell lining of the vessels exposes adhesive proteins within the subendothelial matrix, leading to platelet attachment. Thereafter platelet spreading occurs as well as platelet secretion. The secretion of the content of platelet granules can stimulate circulating platelets to acquire new adhesive properties. Finally, stimulated platelets interact with one another during platelet aggregation and a platelet-rich thrombus is formed, which can compromise the patency of blood vessels. Furthermore activated platelets accelerate the rate at which coagulation proteins are activated: phospholipids on the platelet surface facilitate thrombin generation and fibrin strand formation.

Arterial and venous thrombosis and their complications including ischemic stroke, acute myocardial infarction and venous thromboembolism, represent the major cause of morbidity and mortality in the developed countries.

Prevention and treatment of thrombosis are therefore based on administration of antiplatelet drugs, anticoagulants or thrombolytic therapy or combinations of them.

The pituitary Adenylyl Cyclase Activating Peptide (PACAP 1-38) is a 38-amino acid peptide that was first isolated from ovine hypothalamic extracts on the basis of its ability to stimulate cAMP formation in anterior pituitary cells (1,2). PACAP is part of the vasoactive intestinal polypeptide (VIP)- glucagon- growth hormone releasing factor-secretin superfamily. Its role in biology is probably crucial, since the sequence of PACAP is highly conserved during the evolution from protochordate to mammals. PACAP is widely expressed: in the central and periperal nervous system, the urogenital system, the gastro-intestinal tract, several endocrine glands. Also PACAP receptors are widely distributed (2). Twee classes of PACAP binding sites have been characterized on their relative affinities for PACAP and VIP: type I binding sites with high affinity for PACAP (Kd =0,5nM) and much lower affinity for VIP (Kd > 500nM) and type II binding sites, which are widely distributed in various peripheral organs, characterized with similar affinities for PACAP and VIP (Kd = 1nM). Molecular cloning of PACAP receptors has demonstrated the existence of three distinct receptor subtypes that are abundantly spread in many tissues: the PACAP-specific PAC1 receptor, coupled to different signal transduction systems, and two PACAP: VIP-indifferent VPAC1 and VPAC2 receptors,

which are primarily coupled to adenylyl cyclase. We found that human platelets do express the VPAC1 receptor. The exact biological and pharmacological function of PACAP has presently been investigated in many organs and tissues as in endocrine glands, central nervous system, respiratory system, cardiovascular system and gastrointestinal tract. Although also extensive studies have been performed on its function in the immune system, no data are available concerning its function on haemostasis.

We have recently found that PACAP has an important function in primary haemostasis: platelet number as well as platelet function are highly influenced by PACAP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Brief description of the drawings

Figure 1. *Family presentation.* Squares, male; circles, female; filled symbols, affected individuals; open symbols, unaffected individuals. The proband is indicated with an arrow. Black filled symbols represent members with severe mental retardation and a partial trisomy 18p and monosomy 20p, striped boxes represent members with borderline IQ and the balanced translocation t(18;20) (p21,p13) while question marks stand for members with unexplained mental retardation but unknown karyotype.

Figure 2. *Platelet aggregation and adenylyl cyclase activity.* A. IC_{50} ± accuracy values for indicated individuals and the mean IC_{50} value for Iloprost in the platelet aggregation inhibition test with 2 µg/ml collagen in 22 controls were calculated. A significantly (P 0.03* or P 0.0076**) lower IC_{50} value indicates a Gs hyperfunction. The right column of this table illustrates the significantly decreased response to collagen (µg/ml) for respectively IV:5 and V:3 (P 0.003*) versus V:4 and VI:1 (P 0.0001**) compared to 10 controls or IV:6. EC_{50} is expressed as collagen concentration that induces aggregation with amplitude 50 % of maximal aggregation. B. Measurements of cAMP levels under basal conditions performed in duplicate (left panel) or after stimulation with Iloprost (1 ng/ml) for various time intervals (right panel) in platelets from VI:1 (■) or an unrelated control (*). C. Measurements of cAMP levels under basal conditions performed in

duplicate (left panel) or after stimulation with isoproterenol (1 μM) for various time intervals (right panel) in fibroblasts from VI:1 (■), a patient with trisomy 18 (▲) or an unrelated control (✳). All cAMP measurements were performed in the presence of the phosphodiesterase inhibitor IBMX (400 μM).

Figure 3. *Localization of the PACAP-gene by FISH.* Two color FISH with probe Y841C3 (green) and two centromeric probes for chromosome 18 and 20 (red) for patient VI:1 and his mother V:3. The arrows point to the PACAP signals.

Figure 4. *PACAP detection in fibroblasts and plasma.* A. Semi-quantitative RT-PCR using 20 cycles showed PACAP(1-38) overexpression in fibroblasts from patientVI:1 compared with two controls. β-actin is the internal control. B. PACAP detections by ELISA in plasma from citrate (left panel) or ACD (right panel) blood show pronounced or moderately increased PACAP level in respectively VI:1 (■) and V:4 (▲) or IV:5 (●) and V:3 (◆) versus a citrated plasma pool (✳) or IV:6 (✧). C. Collagen induced aggregation of control platelets in plasma from a control or from patient IV:1 (two experiments shown).

Figure 5. *Role of PACAP(6-38) in platelet aggregation.* A. Dose-dependent stimulatory effect of PACAP(6-38) on collagen-induced (0.2 μg/ml) platelet aggregation. B. The platelet aggregation inhibition test with collagen (2 μg/ml) and different concentrations of Iloprost (ng/ml) as indicated in the absence (left panels) and presence (right panels) of PACAP(6-38) for a control (upper panels) or patient VI:1 (lower panels.

Figure 6. *Effect of anti-PACAP antibodies in mice.* Platelet aggregation was performed in PRP pooled from five mice of each group with 250 x 10^3 plt/μl. A. Stimulatory effect of a polyclonal anti-PACAP antibody (10 μg/ml) on collagen-induced (0.35 μg/ml) platelet aggregation. B. The platelet aggregation inhibition test with collagen (2 μg/ml) and preincubation of Iloprost (10 ng/ml) for mice injected with the indicated antibodies. C. Platelet aggregation induced with a low concentration of collagen (0.2 μg/ml) for mice injected with the indicated antibodies.

Figure 7. The mean platelet number per µl for mice (n=5)injected with either polyclonal anti-PACAP (A) or an irrelevant anti-β2-glycoprotein I (B) antibody was determined 14 days after the first antibody injection.

Figure 8. Mean platelet number +/- SD for mice (n=5) injected with either polyclonal anti-PACAP (A) or anti-vWF (75H4B12) (B) antibody determined at the indicated days.

Figure 9. Mean platelet number/µl +/- SD for mice (n=5) injected with either polyclonal anti-PACAP (A) or an irrelevant anti-XLαs (B) polyclonal antibody determined at the indicated days. Data represent separated experiments A and B.

PACAP overexpression in patients

Patient descriptions.

We describe a family characterised by an unbalanced segregation of the reciprocal translocation t(18,20) (p21,p13), of which different members suffer from unexplained mental retardation (Figure 1). The propositus (VI:1) is a 23-year-old boy with a hypogonadotropic hypogonadism and is followed for epilepsy, severe mental retardation, hyperactive behavior and hypotonia. He has an increased bleeding tendency and the Ivy bleeding time was markedly prolonged (> 15 minutes) but coagulation studies are normal. Electron microscopy of his platelets is completely normal but he presented on different occasions with a moderate thrombocytopenia, as his platelet count is always about 70-90 x 10^3 platelets/µl. His karyotype shows a partial trisomy 18p and monosomy 20p. His brother (VI:2), father (V:2) and maternal grandmother (IV:6) are phenotypically normal, have no bleeding problems and have a normal karyotype. In contrast, his mother (V:3) and maternal grandfather (IV:5) have no obvious neurologic abnormalities but a borderline IQ. They carry the balanced translocation t(18,20) (p21,p13). They don't have any obvious bleeding problems and have a normal platelet count. His 47-year-old uncle (V:4) also suffers from severe mental retardation, pronounced recurrent epistaxis and cryptorchidism. Furthermore, he frequently has gastric bleedings and his platelet count is

around 150 x 10^3 platelets/µl. He also has a partial trisomy 18p and monosomy 20p. Two other family members (IV:2 and V:1) are known with unexplained mental retardation but from these individuals no DNA samples or further clinical information are available.

Adenylyl cyclase activity in platelets and fibroblasts

The propositus VI:1 has disturbed platelet function with a gain-of-Gs activity measured by the platelet aggregation inhibition test, similar to what we described for patients with the XLαs insertion (3,4). Platelets from the patients (VI:1 and V:4) with the partial trisomy 18p/monosomy 20p had a significantly increased sensitivity towards a Gs agonist, the prostacyclin analogue Iloprost (Figure 2A), while platelets from the family members (IV:5 and V:3) with the balanced translocation showed a moderately increased sensitivity. The IC_{50} value for member IV:6 with the normal karyotype is within the rage of the IC_{50} values from 22 unrelated controls.

An important difference between patients from this family and the patients with the XLαs insertion is their decreased sensitivity towards the platelet agonist collagen. The collagen concentration to obtain 50 % aggregation for platelets from V:4 and VI:1 is significantly higher than for platelets from unrelated controls or the normal member IV:6 (Figure 2A). The reactivity of platelets from IV:5 and V:3 towards collagen is again mildly affected.

For patients with the XLαs insertion, we demonstrated that the functional responses mediated by stimulation of Gs agonists are due to hyperactivity of adenylyl cyclase only when Gs-coupled receptors are stimulated (3,4). These patients had normal basal cAMP levels. We hypothesized that in the platelets of the propositus VI:1, adenylyl cyclase is already activated under basal conditions. In addition to an increased cAMP response to Iloprost, patient VI:1 indeed shows higher basal cAMP levels (Figure 2B). We also performed cAMP measurements in fibroblasts from VI:1 and found a similar increased basal and stimulated cAMP response (Figure 2C).

PACAP(1-38) mRNA and protein overexpression

Patient VI:1 has a normal Gsα mRNA and protein expression level and the coding sequence for the Gsα gene and XL-exon1 were completely normal. Since this patient had

a partial trisomy 18p and monosomy 20p, these chromosomes were screened for candidate genes. Interestingly, measurement of the adenylyl cyclase activity in fibroblasts from an unrelated patient with a complete trisomy 18 showed similarly increased basal and stimulated cAMP levels (Figure 2C). The gene for PACAP (*ADCYAP1*) is located on chromosome 18p31-32 (5) and is a possible candidate since its active peptide, PACAP(1-38), stimulates Gs-coupled receptors and thereby activates adenylyl cyclase. FISH analysis with YAC clone Y841C3 (6), that contains *ACDYAP1*, showed that the translocation results in three copies of the gene in patients VI.1 and V:4 (Figure 3).

Human skin fibroblasts express PACAP(1-38) and the PACAP type1-receptor (VPAC1) (7). PACAP(1-38) mRNA was overexpressed in fibroblasts from patient VI:1 (Figure 4A) by semi-quantitative RT-PCR. No PACAP mRNA was found in platelets by RT-PCR, probably due to their unstable RNA. However, we could show by western blot analysis that platelets express the VPAC1 receptor (data not shown). The active peptide PACAP (1-38) is mainly expressed in testis and brain but this peptide can cross the blood-brain barrier and is stably transported in plasma through coupling with ceruloplasmin (8,9). PACAP(1-38) was detected in human plasma by ELISA and significantly higher levels were found in patients VI:1 and V:4, and moderately increased levels in IV:5 and V:3, in contrast to a plasma pool of unrelated controls or IV:6 (Figure 4B). Platelet aggregation using washed control platelets resuspended in citrated plasma from a control or patient VI:1, indicated that plasma from VI:1 inhibits the collagen induced aggregation (Figure 4C). This could be due to the increased amount of PACAP(1-38) in this plasma.

Role for PACAP in platelet aggregation

To determine the effects of the Gs agonist PACAP(1-38) as an inhibitor of collagen induced platelet aggregation, we performed additional aggregations with its antagonist PACAP(6-38). This recombinant peptide has a 10-100 times higher affinity for the VPAC1 receptor than PACAP(1-38) but seems not to activate adenylyl cyclase (2). PACAP(6-38) activates the collagen-induced platelet aggregation in a dose-dependent manner (Figure 5A). In the presence of PACAP(6-38), basal cAMP levels are 10-20 %

lower (data not shown). The functional platelet aggregation inhibition test for a control person in the presence of PACAP (6-38) results in a Gs loss-of-function (Figure 5B). The influence of PACAP(6-38) on the platelet aggregation test for patient VI:1 was not that pronounced, probably because his PACAP plasma levels were too high.

Role for PACAP in platelet aggregation by studies in mice.

Functional platelet studies from patient VI:1 show that increased levels of PACAP(1-38) in plasma result in increased basal cAMP levels and a platelet hypofunction. The role of PACAP(1-38) in platelet function was also studied in mice by subcutaneous injection of polyclonal or monoclonal anti-PACAP antibodies. These antibodies had a similar effect on platelet aggregation as the PACAP antagonist PACAP(6-38). Platelets incubated with anti-PACAP antibodies (10 µg/ml) show an enhanced response towards collagen stimulation (Figure 6A). Moreover, when mice were functionally tested by platelet aggregation 7 days after their last injection, mice treated with anti-PACAP antibodies show the opposite phenotype to that observed in patient VI:1. In contrast to the treatment with the aspecific antibody against β2-glycoprotein I, anti-PACAP-treated mice show a weaker response towards activation of the Gs pathway and have an enhanced response towards collagen stimulation (Figure 6B,C).

Role of PACAP in thrombocytopenia

As the above described patient was also thrombocytopenic, we hypothesized that PACAP(1-38) or increased cAMP levels could lead to a defective megakaryocyte maturation. The role of PACAP(1-38) in thrombopoiesis was further studied in mice by subcutaneous injection of polyclonal or monoclonal anti-PACAP antibodies. The mice injected with the anti-PACAP antibodies (group A, n=5) have furthermore increased platelet numbers in contrast to the control group (group B, n=5) ($1194 \pm 237 \times 10^3$ plt/µl versus $722 \pm 178 \times 10^3$ plt/µl, p= 0.01 - unpaired T-test) (Figure 7).

This experiment was repeated but now the platelet number was determined during the experiment at different time points (days 0, 3, 7, 9, and 14) by tail bleeding (figure 8).

Mice injected with anti-PACAP antibodies (at day 0, 3, and 7) and already show increased platelet numbers 3 days after antibody injection.

We studied the increased thrombopoiesis after pre-treatment with a polyclonal anti-PACAP antibody under conditions of chemically suppressed bone marrow by the agent busulfan. This was done by subcutaneous injection of mice with either a polyclonal anti-PACAP or a control polyclonal antibody (at days 0, 3, and 7) and afterwards an intraperitonal injection of Busulfan (20 mg/kg) (at days 8 and 11). The platelet number was counted at different time points and we found that mice pretreated with the polyclonal anti-PACAP antibody recovered more rapidly from their thrombocytopenic condition than the mice injected with the control antibody (Figure 9A,B).

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2. Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharm Rev* 2000; 52: 269-324.
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5. Hosoya M, Kimura C, Ogi K, Ohkubo S, Miyamoto Y, Kugoh H, Shimizu M, Onda H, Oshimura M, Arimura A, Fujino M. Structure of the human pituitary adenylate

cyclase-activating polypeptide (PACAP) gene. *Biochim Biophys Acta* 1992; 1129: 199-206.

6. Chang E, Welch S, Luna J, Giacalone J, Francke U. Generation of a human chromosome 18-specific YAC clone collection and mapping of 55 unique YACs by FISH and fingerprinting. *Genomics* 1993; 17: 393-402.

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ABSTRACT

The present invention relates to a new method for prevention and treatment of either thrombosis or bleeding based on administration of pituitary adenylyl cyclase activating peptide (PACAP) mimetics or inhibitors respectively or by activating or blocking respectively of a platelet receptor, VPCA1.

Fig 1.

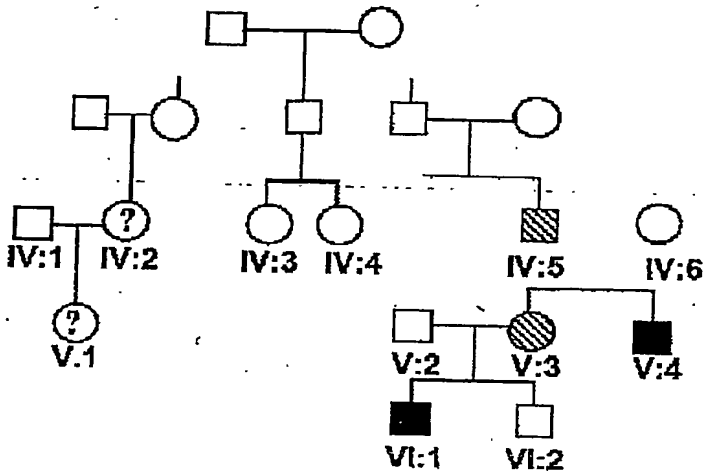
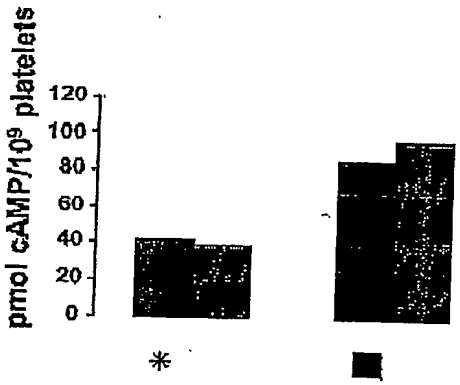


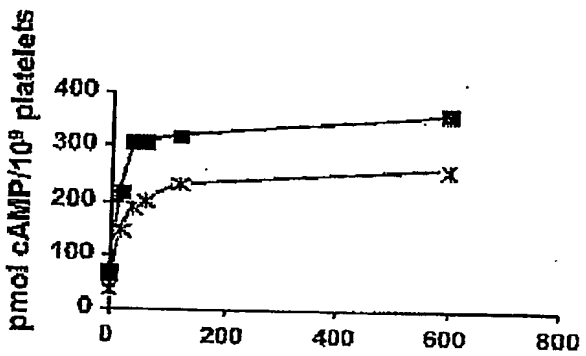
Fig. 2

A

	$IC_{50} \pm SD$ Iloprost	$EC_{50} \pm SD$ Collagen
IV:6	0.96 ± 0.002	0.25 ± 0.012
IV:5	0.50 ± 0.003*	0.74 ± 0.013
V:3	0.47 ± 0.003*	0.75 ± 0.006
V:4	0.27 ± 0.005**	1.08 ±0.006
VI:1	0.34 ± 0.004**	1.03 ± 0.008
Controls	1.04 ± 0.39 (n = 22)	0.22 ± 0.6 (n = 10)

B





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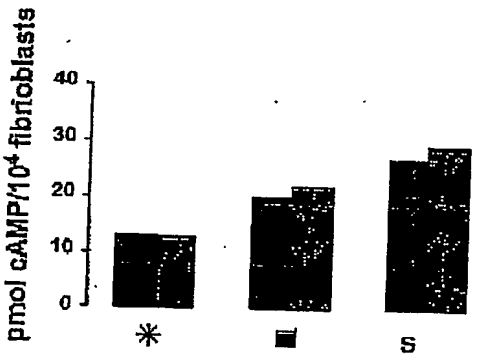


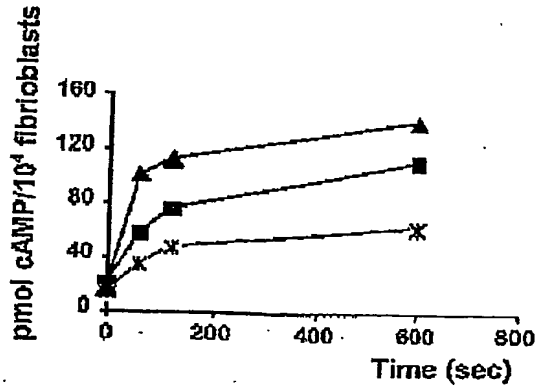
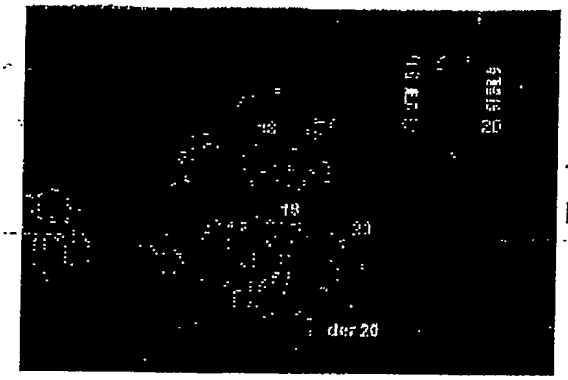
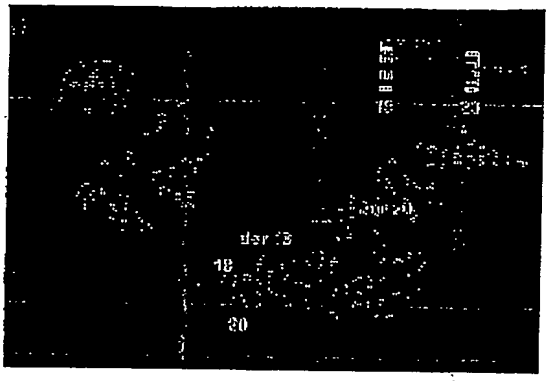


Fig. 3.



VI:1
Trisomy 18p21
Monosomy 20p13



V:3
Balanced translocation
t(18,20) (p11.21,p13)

Fig 4

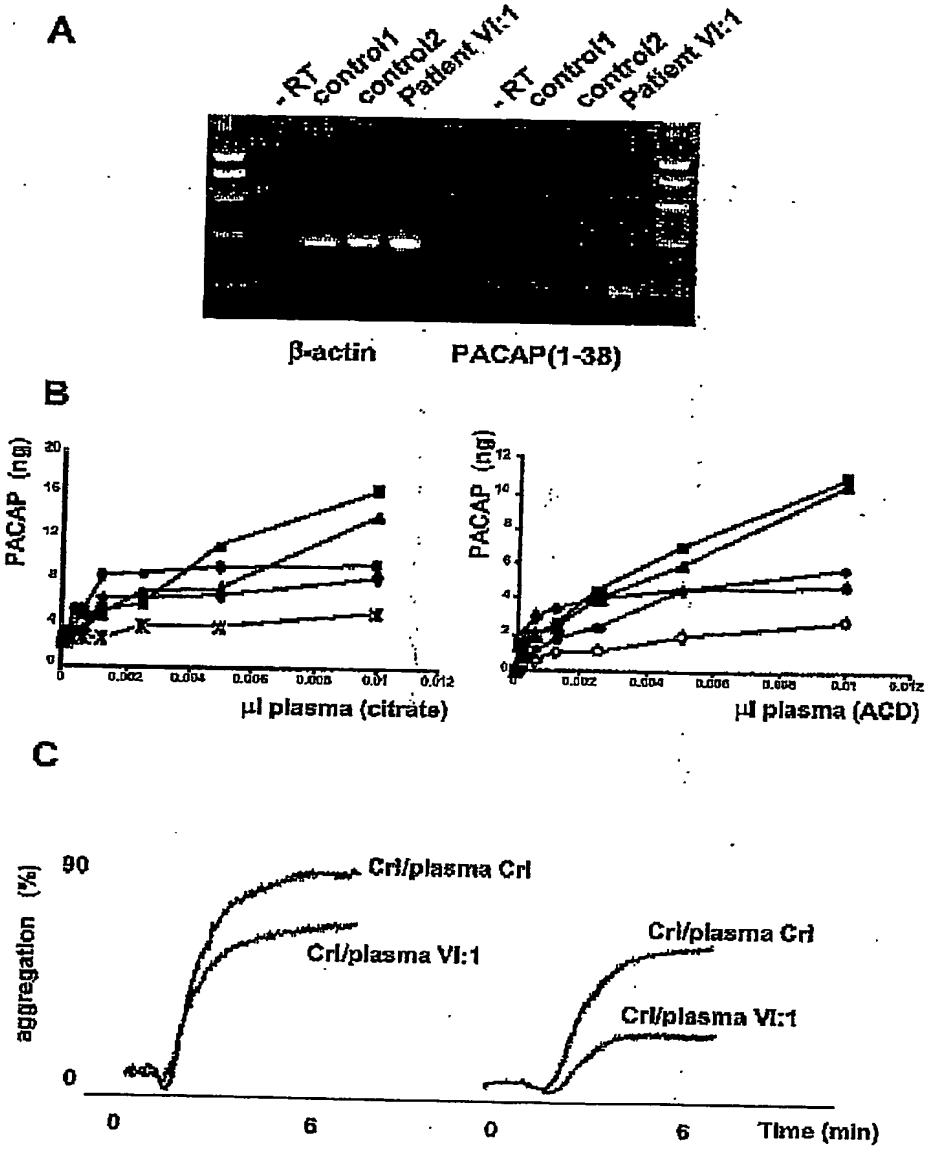


Fig. 5.

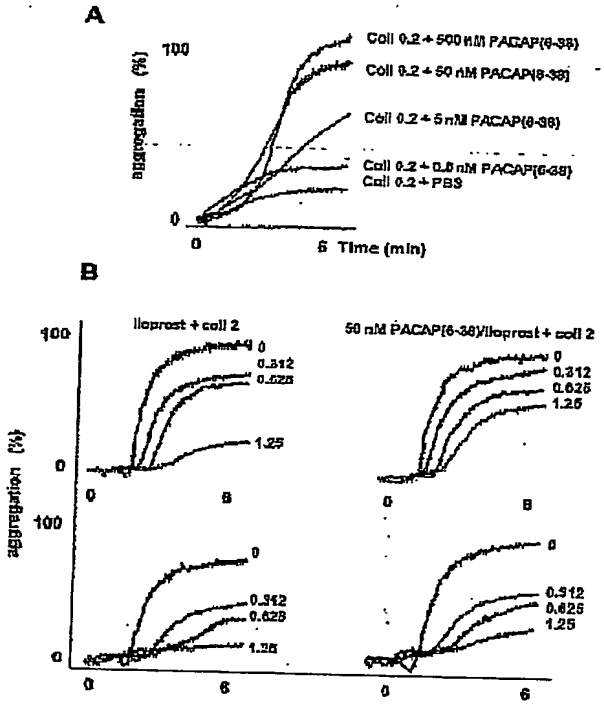
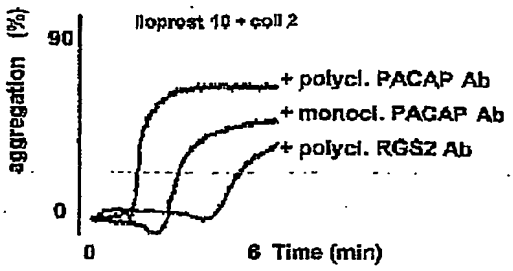


Fig. 6

A



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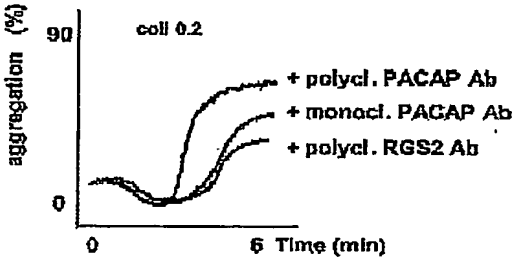


C

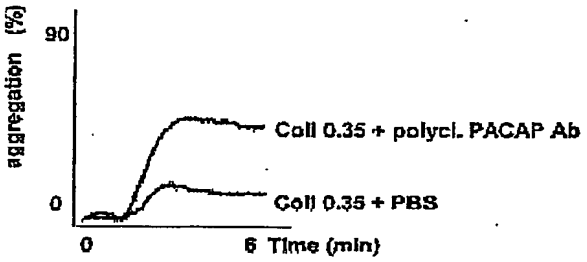
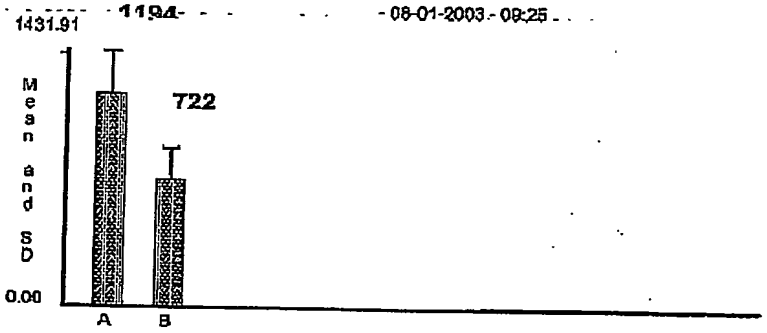


Fig. 7



P = 0.01

Fig. 8

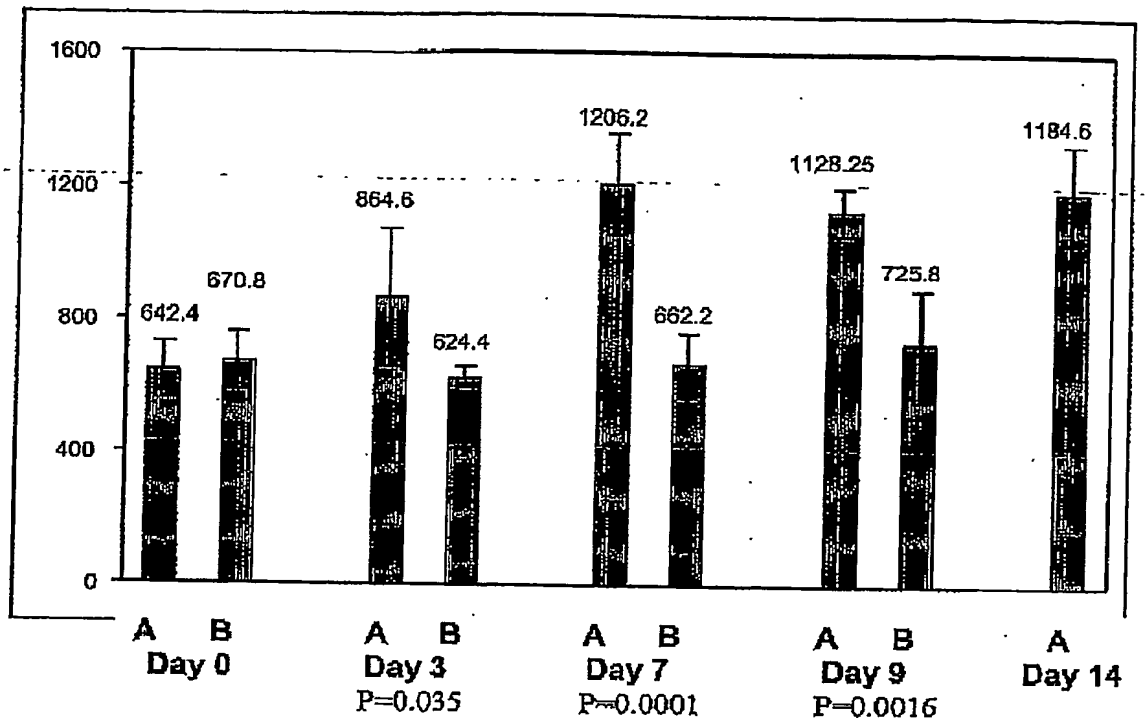
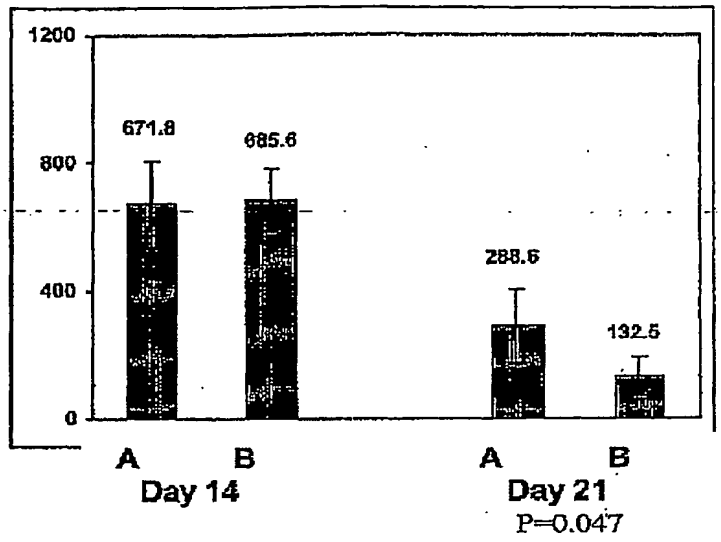
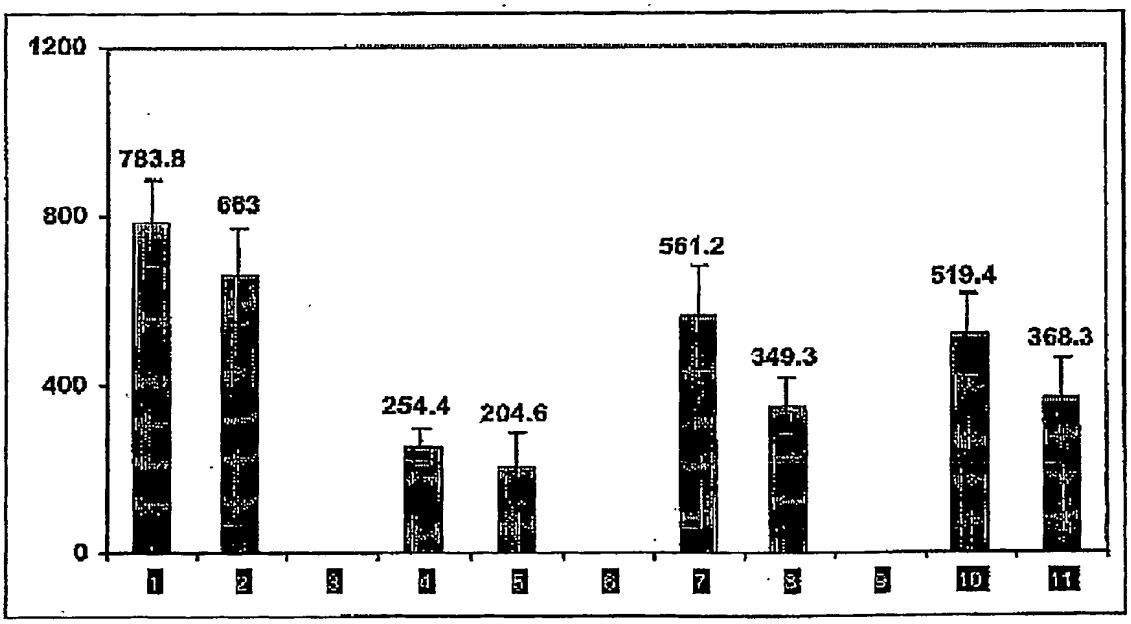


Fig. 9



P=0.047



A B Day 14 | A B Day 21 P=0.27 | A B Day 29 P=0.03 | A B Day 31 P=0.05